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[Address or Domicile] 303 Akurejidennsu Hokoutyou 1-13-9 Hokoutyou, Matsuyama-shi, Ehime

[Name] Tatsuya Sawasaki

[Address or Domicile] 701-3-1-8 Hontyou, Matsuyama-shi, Ehime

[Name] Tatsuya Sawasaki

[Address or Domicile] 478-17 Kumanodai, Matsuyama-shi, Ehime

[Name] Yaeta Endo

[Applicant for Patent]

[Identification Number] 594016182

[Name or Nomenclature] Yaeta Endo

[Applicant for Patent]

[Identification Number] 000005968

[Name or Nomenclature] Mitsubishi Chemical Corporation

[Attorney]

[Identification Number] 100103997

[Patent Attorney]

[Name or Nomenclature] Satoshi Hasegawa

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[List of Documents]

[Matter]	Specification	1
[Matter]	Drawing	1
[Matter]	Abstract of the Disclosure	1

CLAIMS

【claim 1】

A labeled single chain antibody comprising having a structure in which a heavy chain and a light chain of the antibody are crosslinked through a linker and comprising carrying a labeling substance in the linker part.

【claim 2】

The antibody of claim 1, wherein the heavy chain and the light chain of the antibody are variable regions.

【claim 3】

The antibody of claim 1 or 2, wherein the labeling substance is a substance that is capable of binding to a polypeptide of the linker part of the antibody in the presence of a specific enzyme.

【claim 4】

The antibody of claim 1 or 2, wherein the labeling substance is incorporated as one part of the linker part of the antibody.

【claim 5】

The antibody of claim 3, wherein the labeling substance is biotin and the enzyme is a biotin ligase.

【claim 6】

A DNA, wherein DNAs encoding a heavy chain and a light chain of an antibody having binding ability against a specific antigen are linked through a DNA encoding a linker.

【claim 7】

The DNA of claim 6, wherein the heavy chain and the light chain of the antibody are variable regions.

【claim 8】

The DNA of claim 6 or 7, wherein the DNA encoding a linker comprises a nucleotide sequence that is capable of binding with a labeling substance in the presence of a specific enzyme after translation.

【claim 9】

The DNA of claim 6 or 7, wherein the DNA encoding a linker

comprises a nucleotide sequence encoding a labeling substance.

【claim 10】

The DNA of claim 8, wherein the nucleotide sequence that is capable of binding with a labeling substance is birA.

【claim 11】

A method for producing a labeled single chain antibody, wherein the DNA according to any of claim 6 to 8 or 10 is subject to transcription and translation using a protein synthesis system in the presence of a labeling substance and a specific enzyme.

【claim 12】

A method for producing a labeled single chain antibody, wherein the DNA according to claim 6,7 or 9 is subject to transcription and translation using a protein synthesis system.

【claim 13】

The method for producing a labeled single chain antibody according to claim 11 or 12, wherein the protein synthesis system is a cell-free protein translation system, and a concentration of a reducing agent in a translation reaction solution thereof is a concentration whereby a disulfide bond of the antibody to be produced is maintained and cell-free protein synthesis is enabled.

【claim 14】

The method of claim 13, wherein the method is conducted in the presence of an enzyme that catalyzes a disulfide bond exchange reaction.

【claim 15】

A method for producing an immobilized single chain antibody, wherein the antibody according to any of claims 1 to 5 is brought into contact with a reaction plate compartmentalized into a plurality of regions having on the surface thereof a substance that binds specifically with a labeling substance of the antibody:

【claim 16】

The method of claim 15, wherein the labeling substance

is biotin and the substance that binds specifically with the labeling substance is streptavidin.

【claim 17】

An immobilized single chain antibody prepared by the method according to any one of claim 15 or 16.

【claim 18】

A method for analyzing an antigen-antibody reaction, wherein a test substance is brought into contact with the immobilized single chain antibody of claim 17, and binding ability of the test substance against the immobilized single chain antibody is analyzed.

SPECIFICATION

【Title of invention】 A labeled antibody and use thereof

【Detailed description of the invention】

【0001】

【Field of the Invention】

The present invention relates to a single chain antibody having a structure in which a heavy chain and a light chain of the antibody are crosslinked through a linker, and the linker part having a labeling substance, and methods for utilizing the same.

【0002】

【Description of the Related Art】

A single chain antibody is small in size in comparison to a complete IgG since it comprises only an antigen-binding region, and thus a feature thereof is that non-specific binding to a cell can be lessened. When using a single chain antibody for analysis of an antigen-antibody reaction, a method has been developed in which various labels are attached to antibodies for the purpose of tracking immunoreaction (Cloutier, S. M. et al., Mol. Immunol., 37, 1067-1077 (2000)). Although various methods have been proposed for labeling an antibody, such as a method in which biotin or the like is bound to the C terminus or N terminus of the antibody using a biotin ligase (Cloutier, S. M. et al., Mol. Immunol., 37, 1067-1077 (2000)), a problem has existed in that the activity of the antibody to bind with an antigen is reduced by the label.

【0003】

In recent years, the development of techniques for immobilizing this kind of antibody on chips or beads or the like for the purpose of detecting specific antigens present on a cell surface rapidly and in large amounts has also been remarkable (Mitchell, P., Nature Biotechnology, 20, 225-229 (2002)). More specifically, while techniques such as microspotting, microprinting, and chemical modification are

used, each of these has problems that the binding activity of the antibody to an antigen is lowered, the high-density application is difficult, and the like.

【0004】

Meanwhile, a method has also been proposed in which substances having specific binding ability such as streptavidin/biotin that covalently bind to immobilized protein reaction plates are bonded as linkers. However, in these methods also, no examples exist in which an immobilized antibody maintained its binding ability against the antigen.

【0005】

【Problems to be resolved by the invention】

It is an object of this invention to provide a method for labeling an antibody while maintaining the binding ability of the antibody against its antigen. A further object of this invention is to provide a method for immobilizing an antibody while maintaining the binding ability of the antibody against its antigen and a labeled single chain antibody for use in the method.

【0006】

【Means of solving the problems】

After conducting concentrated research to solve the above-described problems, the present inventors bound biotin to the linker part of a single chain antibody in which the heavy chain and the light chain of the antibody were connected through a linker, and brought the single chain antibody into contact with a reaction plate whose surface was coated with streptavidin to bind the antibody to the reaction plate. When we brought an antigen into contact with the immobilized single chain antibody produced in this manner, we found that the binding ability of the antibody against the antigen was maintained at an extremely high level. This invention was accomplished based on these findings.

【0007】

More specifically, the present invention provides the following:

1.A labeled single chain antibody comprising having a structure in which a heavy chain and a light chain of the antibody are crosslinked through a linker and comprising carrying a labeling substance in the linker part.

2.The antibody of preceding 1, wherein the heavy chain and the light chain of the antibody are variable regions.

3.The antibody of preceding 1 or 2, wherein the labeling substance is a substance that is capable of binding to a polypeptide of the linker part of the antibody in the presence of a specific enzyme.

4.The antibody of preceding 1 or 2, wherein the labeling substance is incorporated as one part of the linker part of the antibody.

5.The antibody of preceding 3, wherein the labeling substance is biotin and the enzyme is a biotin ligase.

6.A DNA, wherein DNAs encoding a heavy chain and a light chain of an antibody having binding ability against a specific antigen are linked through a DNA encoding a linker.

7.The DNA of preceding 6, wherein the heavy chain and the light chain of the antibody are variable regions.

8.The DNA of preceding 6 or 7, wherein the DNA encoding a linker comprises a nucleotide sequence that is capable of binding with a labeling substance in the presence of a specific enzyme after translation.

9.The DNA of preceding 6 or 7, wherein the DNA encoding a linker comprises a nucleotide sequence encoding a labeling substance.

10.The DNA of preceding 8, wherein the nucleotide sequence that is capable of binding with a labeling substance is birA.

11.A method for producing a labeled single chain antibody, wherein the DNA according to any of preceding 6 to 8 or 10 is subject to transcription and translation using a protein synthesis system in the presence of a labeling substance and a specific enzyme.

12.A method for producing a labeled single chain

antibody, wherein the DNA according to preceding 6,7 or 9 is subject to transcription and translation using a protein synthesis system.

13.The method for producing a labeled single chain antibody according to preceding 11 or 12, wherein the protein synthesis system is a cell-free protein translation system, and a concentration of a reducing agent in a translation reaction solution thereof is a concentration whereby a disulfide bond of the antibody to be produced is maintained and cell-free protein synthesis is enabled.

14.The method of preceding 13, wherein the method is conducted in the presence of an enzyme that catalyzes a disulfide bond exchange reaction.

15.A method for producing an immobilized single chain antibody, wherein the antibody according to any of precedings 1 to 5 is brought into contact with a reaction plate compartmentalized into a plurality of regions having on the surface thereof a substance that binds specifically with a labeling substance of the antibody:

16.The method of preceding 15, wherein the labeling substance is biotin and the substance that binds specifically with the labeling substance is streptavidin.

17.An immobilized single chain antibody prepared by the method according to any one of preceding 15 or 16.

18.A method for analyzing an antigen-antibody reaction, wherein a test substance is brought into contact with the immobilized single chain antibody of preceding 17, and binding ability of the test substance against the immobilized single chain antibody is analyzed.

【0008】

【Embodiments of the present invention】

(1) Labeled single chain antibody

A single chain antibody used in this invention may be any kind of substance, as long as it is a substance in which a heavy chain and a light chain of an antibody are connected through a linker and which has activity for binding with an antigen for

which the antibody has specific binding affinity. Preferably, the substance used is one in which the heavy chain of an antibody is positioned at the N terminus of the single chain antibody molecule. As an antibody, a monoclonal antibody having activity that recognizes and binds with a specific antigen is preferable. Further, with respect to a heavy chain and light chain of an antibody, it is not necessary that the substance comprise the full length thereof, as long as the substance comprises a part that is sufficient for recognizing an antigen and for having specific binding affinity thereto. More specifically, a variable region is preferably used.

【0009】

A linker is not particularly limited, as long as it has a length that is sufficient for a heavy chain and a light chain of an antibody to be crosslinked through the linker, and also has a structure for having a labeling substance. In general, a polypeptide comprising 10 to 30 amino acids is preferably used. A specific structure can be suitably selected in accordance with a labeling substance that is described hereunder.

【0010】

As a labeling substance, a substance that can be used for the purpose of labelling the single chain antibody of this invention (hereunder, this is sometimes referred to as "signal substance") and a substance that can be used for the purpose of immobilizing the single chain antibody of this invention (hereunder, this is sometimes referred to as "immobilizing substance") are preferable. More specifically, examples of a signal substance include a fluorescent dye that is capable of binding to an amino acid, such as a dye belonging to fluorescein, rhodamine, eosin, or NBD; a photosensitizer, such as methylene blue or rose bengal; or a substance that imparts a specific signal in nuclear magnetic resonance (NMR), for example an amino acid comprising a fluorine or phosphorus atom. As an immobilizing substance (hereunder, this is sometimes referred to as "adapter substance"), any substance may be used as long as it is a substance that binds with a specific substance that

has been bound to a solid phase surface. Examples of a combination of an immobilizing substance and an adapter substance include various types of receptor proteins and a ligand thereof, such as biotin and a biotin-binding protein such as avidin or streptavidin; maltose and a maltose-binding protein; guanine nucleotide and G protein; a polyhistidine peptide and a metal ion such as nickel or cobalt; glutathione-S-transferase and glutathione; a DNA-binding protein and a DNA; an antibody and an antigen molecule (epitope); calmodulin and a calmodulin-binding peptide; ATP-binding protein and ATP; or estradiol receptor protein and estradiol. Either of these substances may the immobilizing substance or the adapter substance. Among them, preferably biotin is used as an immobilizing substance and streptavidin as an adapter substance, or a polyhistidine peptide is used as an immobilizing substance and nickel or the like is used as an adapter substance.

【0011】

A substance that is capable of binding to a polypeptide of a linker part of an antibody in the presence of a specific enzyme in a method for binding a substance to the linker part can also be used as a labeling substance. Examples of this type of substance include biotin and the like. When using biotin as a labeling substance, examples of a specific enzyme include a biotin ligase, and examples of a linker include a substance having an amino acid sequence that can be recognized by a biotin ligase.

【0012】

Further, a labeling substance may be a substance that is incorporated as one part of a linker part of an antibody, and as a specific example thereof a polyhistidine peptide may be mentioned. In this case, a substance comprising a polyhistidine peptide may be used as a linker.

Binding of a labeling substance to a linker part, or incorporation a labeling substance therein, can be carried out according to a known method in accordance with the signal

substance to be used or the properties of the immobilizing substance and adapter substance.

【0013】

(2) Method for producing labeled single chain antibody

A labeled single chain antibody of this invention can be produced, for example, according to the methods described below. First, (i) a monoclonal antibody that recognizes a protein of interest or a part thereof as an antigen is prepared, and (ii) DNA encoding the monoclonal antibody is acquired. Then, the sequences encoding the heavy chain and light chain thereof are identified, and these are linked together sandwiching a nucleotide sequence encoding the linker (hereunder, this DNA fragment may sometimes be referred to as "single chain antibody unit"). (iii) The protein that is encoded by the thus-produced single chain antibody unit is then synthesized by a suitable method that properly maintains the structure thereof. In the case of binding a labeling substance to the linker part at the time of synthesis or after synthesis, the appropriate binding procedure is conducted. These methods are described in detail hereunder.

【0014】

(i) Preparation of monoclonal antibody

An antigen of the single chain antibody of this invention is not particularly limited, and may be any substance as long as the substance has immunogenicity. More specifically, for example, a sugar chain of Salmonella or the like may be mentioned. A known method conventionally used in the art can be used as a method of preparing a monoclonal antibody that specifically recognizes these antigens, and for a polypeptide used as an antigen, a sequence that is suitable as an epitope (antigenic determinant) with high antigenicity can be selected in accordance with a known method and used. As a method of selecting an epitope, for example, commercially available software such as Epitope Adviser (manufactured by Fujitsu Kyushu System Engineering) or the like can be used.

【0015】

As a polypeptide used as the aforementioned antigen, a synthetic peptide that was synthesized in accordance with a known method is preferably used. Although a polypeptide to be used as an antigen may be prepared in an appropriate solution or the like in accordance with a known method and then used to immunize a mammal such as rabbit or mouse, in order to conduct stable immunization and raise the antibody titer, immunization is preferably conducted using an antigen peptide that forms a conjugate with a suitable carrier protein, with the addition of an adjuvant or the like.

【0016】

The route of administration of an antigen at the time of immunization is not particularly limited, and for example, a subcutaneous, intraperitoneal, intravenous or intramuscular route may be used. More specifically, for example, a method may be used in which BALB/c mice are inoculated with an antigen polypeptide several times at intervals of several days to several weeks. Regarding intake of the antigen, while an intake of from 0.3 to 0.5 mg/per inoculation is preferable when the antigen is a polypeptide, the intake can be appropriately adjusted in accordance with the kind of polypeptide and the species of animal to be immunized.

【0017】

After immunization, blood is tentatively collected as appropriate to verify an increase in antibody titer by a method such as enzyme-linked immunosorbent assay (hereunder, this is sometimes referred to as "ELISA") or Western blotting, and blood is then collected from an animal in which the antibody titer has increased sufficiently. By subjecting the obtained blood to a suitable process used in preparation of an antibody, a polyclonal antibody can be obtained. More specifically, for example, a method may be mentioned in which purified antibody is acquired by purifying the antibody component from serum in accordance with a known method. A monoclonal antibody can also be produced using a hybridoma produced by fusing myeloma cell and spleen cell of the animal in accordance with a known method

(Milstein et al., Nature, 256, 495 (1975)). A monoclonal antibody can be acquired, for example, by the method described below.

【0018】

First, antibody-forming cells are acquired from the aforementioned animal in which the antibody titer was raised by immunization of an antigen. Antibody-forming cells are the plasma cells and the precursor cells thereof, lymphoid cells, and while they may be acquired from any part of the individual, they are preferably acquired from the spleen, lymph node, peripheral blood or the like. As myeloma cells to be fused with these cells, in general, an established cell line acquired from mouse, such as 8-azaguanine resistant mouse (derived from BALB/c or the like) myeloma cell line P3X63-Ag 8.653 (ATCC: CRL-1580) or P3-NS1/1Ag 4.1 (Riken Cell Bank: RCB0095) or the like are preferably used. Fusion of the cells can be carried out by mixing the antibody-forming cells and myeloma cells at an appropriate ratio using a suitable cell fusion medium, for example, RPMI 1640 or Iscove's modification of Dulbecco's medium (IMDM), or a medium in which 50% polyethyleneglycol is dissolved in Dulbecco's modified Eagle's medium (DMEM) or the like. Fusion of cells can also be conducted by an electrofusion method (U. Zimmermann et al., Naturwissenschaften, 68, 577 (1981)).

【0019】

A hybridoma can be selected by utilizing the fact that the myeloma cell line used is an 8-azaguanine resistant line and culturing for an appropriate time at 37 °C with 5% CO_2 in a normal culture medium containing a suitable amount of hypoxanthine amino-pterin thymidine (HAT) solution (HAT culture medium). The selection method can be suitably selected and used in accordance with the myeloma cell line used. A monoclonal antibody can be obtained by analyzing according to the aforementioned method antibody titers of antibodies produced by selected hybridomas, isolating a hybridoma producing an antibody having a high antibody titer by a limiting

dilution method or the like, and purifying the monoclonal antibody from culture supernatant obtained by culturing the isolated fused cell in a suitable medium, by an appropriate method such as ammonium sulfate fractionation or affinity chromatography. A commercially available monoclonal antibody purification kit can also be used to purify the monoclonal antibody. Further, peritoneal fluid containing a large quantity of the monoclonal antibody of this invention can also be obtained by allowing the antibody-producing hybridoma obtained in the manner described above to proliferate intraperitoneally in an animal of the same family as the immunized animal or in nude mice or the like.

【0020】

(ii) Acquisition of DNAs encoding heavy chain and light chain of monoclonal antibody and preparation of single chain antibody unit

As a specific method for acquiring DNAs encoding the heavy chain (H chain) and the light chain (L chain) of the monoclonal antibody acquired in the above (i), a method may be mentioned in which the amino acid sequences of one part of the L chain and H chain of immunoglobulin obtained from a hybridoma producing the monoclonal antibody, preferably, parts of the amino acid sequences having a variable region (V region), are analyzed, and on the basis of the amino acid sequences the gene encoding it is cloned. Here, a variable region of the L chain and the H chain of the monoclonal antibody is preferably a region comprising a framework region (FR) and a hypervariable region (CDR).

【0021】

As DNA encoding a variable region of a H chain and L chain obtained in this manner, for example, DNA comprising the sequence described in Anand, N. N., et al., J. Biol. Chem., 266, 21874-21879 (1991) and the like may be mentioned as DNA of a single chain antibody that recognizes O-antigen of Salmonella.

A single chain antibody unit can be prepared by sandwiching DNA encoding a linker between the thus-obtained

DNAs encoding the variable regions of the H chain and L chain, and connecting the two DNA fragments by an appropriate method. In this case, it is not necessary to obtain the single chain antibody unit separately as a DNA fragment, and it may be constructed at the same time as insertion into an expression vector or the like as described later. As DNA encoding a linker, any substance may be used as long as it is DNA encoding a linker as described in (1). More specifically, for example, DNA encoding a linker that includes an amino acid sequence that is recognized by a biotin ligase (birA) (Peter J. Schatz (1993), Biotechnology, 11 (1138-1143)) is preferable, and examples thereof include the substance represented by SEQ ID NO: 1. Further, as an example in which a labeling substance is incorporated as one part of the linker part, a substance comprising a nucleotide sequence encoding a polyhistidine peptide or the like may be mentioned.

【0022】

DNA encoding a linker can be produced using a method used conventionally, and the DNA is preferably produced by chemical synthesis.

【0023】

(iii) Production of single chain antibody

A single chain antibody can be produced by connecting the thus-obtained single chain antibody unit to a suitable promoter to be under the control thereof, and introducing this into a host, or by conducting transcription by an appropriate method and then expressing the single chain antibody under conditions which retain a disulfide bond of the single chain antibody to be produced using a cell-free protein translation system. An antibody having low binding ability against an antigen can also be acquired as an antibody having higher binding ability by use of a known evolutionary engineering technique.

【0024】

A suitable promoter can be appropriately selected in accordance with a host to be used or the RNA synthetase used in transcription. More specifically, when using SP6 RNA

synthetase for transcription, SP6 promoter is preferably used. In the cell-free protein translation system, a base sequence that augments translational activity is preferably inserted between the promoter and the single chain antibody unit. Specific examples of known base sequences that augment translational activity include the 5'-cap structure (Shatkin, Cell, 9, 645- (1976)), Kozak sequence (Kizak, Nucleic Acid Res., 12, 857- (1984)) and the like in eucaryotes, and Shine-Dalgarno sequence and the like in prokaryotes. Further, it has been found that translation promoting activity is also present in the 5'-nontranslated leader sequences of RNA virus (Japanese Patent No. 2814433), and a method has been developed which efficiently conducts protein synthesis using these sequences (Japanese Patent Laid-Open No. 10-146197). In addition, with respect to a random sequence, a sequence obtained by a method that selects a translation enhancer sequence by taking influence on polysome formation as an indicator may also be mentioned (specification of Japanese Patent Application No. 2001-396941). Hereunder, DNA produced in this manner may sometimes be referred to as "translation template."

【0025】

As a specific example of a translation template, a substance having the structure shown in FIG. 1 may be mentioned as an example of a substance recognizing O-antigen of Salmonella.

As a host into which a translation template is introduced, protein synthesis system that can be used in normal protein synthesis and which is capable of retaining a disulfide bond of a single chain antibody is used. As specific example of host, E. coli DHBA strain (Paola Jurado et al. J. Mol. Biol., 320, 1-10(2002)), etc. is exemplified. Also, when a single antibody is prepared in cell-free protein synthesis after transcription of translate template, every extract of cell-free protein synthesis is used so far as it has ability of protein synthesis. As specific example of cell extract is used in this invention, microorganism such as E.coli, embryo of plant seed and cell

extract such as rabbit reticulocyte are exemplified. These cell extracts are used commercialized products or prepared by conventional method. Specifically, E. coli extract is prepared according to method described in Pratt, J. M. et al., Transcription and Translation, Hames, 179-209, B.D. & Higgins, S. J., eds), IRL Press, Oxford (1984).

【0026】

As cell extract of commercialized products, E. coli S30 extract system (Promega) and RTS500 Rapid Translation System (Roche) and etc. are exemplified as extract derived from E. coli extract. Rabbit Reticulocyte Lysate System (Promega) etc. is used as extract derived from rabbit reticulocyte. PROTEISO ™ (Toyobo Co., Ltd.) is used as extract derived from wheat embryo extract.

【0027】

Further, a reaction solution which can retain an intramolecular disulfide bond and also synthesize a protein can be prepared by adjusting the concentration of a reducing agent among the ingredients necessary for protein synthesis of a reaction solution of the above cell-free translation system (hereunder, this is sometimes referred to as "weak reductive translation reaction solution"). Examples of a specific reducing agent and the concentration thereof include dithiothreitol (hereunder, sometimes referred to as "DTT") at a final concentration of 20 to 70 μM, preferably 30 to 50 μM, 2-mercaptoethanol at a final concentration of 0.1 to 0.2 mM, and glutathione/oxidized glutathione at a final concentration within a range of 30 to 50μM/1 to 5 μM.

【0028】

The concentration of a reducing agent in the translation reaction solution is not limited to the above concentrations, and may be suitably modified in accordance with the protein or kind of cell-free protein synthesis translation system to be synthesized. While a method for selecting the range of optimal concentration of a reducing agent is not particularly limited, for example, a method in which assessment is made based on the

effect of an enzyme catalyzing a disulfide bond exchange reaction may be mentioned. More specifically, translation reaction solutions in which the concentration of a reducing agent is variously adjusted are prepared, and an enzyme that catalyzes a disulfide bond exchange reaction is added to these solutions, to conduct synthesis of a protein having an intramolecular disulfide bond. As a control experiment, protein synthesis is carried out in a similar manner in the same translation reaction solutions without adding the enzyme that catalyzes a disulfide bond exchange reaction. A solubilized component of the protein synthesized in the above manner is then isolated by, for example, a method such as centrifugation. A reaction solution in which the solubilized component constitutes 50% (solubilization ratio 50%) or more of the total volume and in which the solubilized component increased after addition of the enzyme that catalyzes a disulfide bond exchange reaction can be judged as suitable as a reaction solution that conducts synthesis while retaining an intramolecular disulfide bond of the protein in its original state. Further, of the ranges of concentration of a reducing agent that was selected on the basis of the aforementioned effect of an enzyme that catalyzes a disulfide bond exchange reaction, concentrations of a reducing agent that generate the largest amount of synthesized protein can be selected as a further preferable concentration range.

【0029】

Methods that can be used to prepare a reaction solution having the aforementioned reducing agent concentration include a method in which cell extract for cell-free protein synthesis that does not include a reducing agent is prepared, and then ingredients required for a cell-free protein translation system are added thereto together with a reducing agent at a concentration within the above concentration range, and a method in which a reducing agent is removed from cell extract for cell-free protein synthesis such that the concentration of the reducing agent is within the aforementioned concentration

range. Since cell extract for cell-free protein synthesis requires advanced reducing conditions when extracting, a method in which a reducing agent is removed from this solution after extraction is more convenient. As a method for removing a reducing agent from cell extract, a method using a carrier for gel filtration and the like may be mentioned. More specifically, for example, a method in which a Sephadex G-25 column is previously equilibrated with a suitable buffer solution that does not include a reducing agent, and cell extract is then passed therethrough may be mentioned.

【0030】

Further, the cell extract may also be used after forming the cell extract into a lyophilized product by lyophilizing, and adding a suitable buffer solution thereto. Preferably the total concentration of a deliquescent substance is made 60 mM or less when lyophilizing. Lyophillization can also be conducted after adding the aforementioned translation template to the cell extract.

Further, by carrying out a translation reaction in which an enzyme that catalyzes a disulfide bond exchange reaction is further added to a weak reductive translation reaction solution, it is possible to conduct highly efficient synthesis of a protein that retains an intramolecular disulfide bond. As an enzyme that catalyzes a disulfide bond exchange reaction, for example, protein disulfide isomerase or the like may be mentioned. The amount of these enzymes to be added to a cell-free translation system can be suitably selected in accordance with the kind of enzyme. More specifically, when adding protein disulfide isomerase to a translation reaction solution that is cell extract for cell-free protein synthesis extracted from wheat embryo, which contains as a reducing agent 20 to 70 μM of DTT, and preferably 30 to 50 μM thereof, the protein disulfide isomerase is added to bring to a final concentration within the range of 0.01 to 10 μM, and preferably 0.5 μM. With respect to the stage for adding an enzyme, from the viewpoint of efficiency of disulfide bond formation, the enzyme is

preferably added prior to the start of the cell-free translation reaction.

【0031】

These cell-free protein translation systems, use of wheat embryo extract is particularly preferable, and a method for producing a single chain antibody will be explained in detail below taking as an example a case using this cell extract.

Examples of cell-free protein translation systems derived from seed of plants other than wheat include those derived from gramineous plants such as barley, rice and corn.

【0032】

As a method for selecting wheat embryo, for example, the method of Johnston, F. B., et al., Nature, 179, 160-161 (1957) can be used, and as a method for producing cell extract from the embryo, a method described in Erickson, A. H., et al. Meth. In Enzymol., 96, 38-50 (1996) or the like can be used. Further, preferable preparation method is as follows.

【0033】

Ordinarily, the embryo component is extremely small and therefore, in order to obtain the embryo in an efficient manner, it is preferable that components other than embryo be removed to as great an extent as is possible. Normally, mechanical force is first applied to the plant seeds so as to produce a mixture comprising embryo, crushed endosperm and crushed seed coat. The crushed endosperm, crushed seed coat and the like are removed from this mixture, so as to produce a crude embryo fraction (a mixture primarily composed of embryo but also containing crushed endosperm and crushed seed coat). It suffices that the force applied to the plant seed be of a strength sufficient to separate the embryo from the plant seed. Specifically, known grinding equipment is used to grind the plant seeds, so as to produce a mixture containing embryo, crushed endosperm and crushed seed coat.

【0034】

The plant seeds can be ground using commonly known grinding apparatus but it is preferable to use grinding

apparatus of the type that applies impact force to the material that is ground, such as a pin mill or a hammer mill. The degree of grinding maybe suitably chosen according to the size of the embryo of the plant seed that is used. For example, wheat grain is usually ground to a maximum length of no greater than 4 mm, and is preferably ground maximum length of no greater than 2 mm. Furthermore, it is preferable that the grinding be performed as dry grinding.

【0035】

Next, a crude embryo fraction is recovered from the ground plant seed produced, using the well-known classifier, such as a sieve. For example, in the case of wheat grain, a crude embryo fraction is recovered using a mesh sieve of a 0.5 to 2.0 mm, and preferably 0.7 to 1.4 mm. Furthermore, if necessary, the seed coat, the endosperm, dust and the like contained in the crude embryo fraction produced can be removed by wind force or electrostatic force.

【0036】

It is also possible to produce a crude embryo fraction using methods that make use of the difference in the specific gravities of embryo, seed coat and endosperm, such as heavy media separation. In order to obtain a crude embryo fraction containing a greater quantity of embryo, a plurality of the methods described above may be combined. Furthermore, it is possible to select the embryo from the crude embryo fraction produced, for example, either visually or using a color sorter, or the like.

As an endosperm component may adhere to the embryo fraction produced in this manner, it is normally preferable that this be washed in order to purify the embryo. It is preferable that this be washed by dispersing/suspending the embryo fraction in cold water or a cold aqueous solution at a temperature that is normally no greater than 10°C and preferably no greater than 4°C and washed until the washing solution is no longer clouded. It is more preferable that the embryo fraction be dispersed/suspended in an aqueous solution

containing a surfactant, which is normally at a temperature of no more than 10°C and preferably at a temperature of no more than 4°C, and washed until the washing solution is no longer clouded. It is preferable that the surfactant be nonionic, and a wide variety of surfactants can be used so long as these are nonionic. Specific examples of suitable substances include Brij, Triton, Nonidet P40, Tween, and the like, which are polyoxyethylene derivatives. From among these, Nonidet P40 is the most suitable. These nonionic surfactants can be used at concentrations sufficient to remove the endosperm component but which do not negatively impact the protein synthesis activity of the embryo component. For example, a concentration of 0.5% can be used. The washing treatment may be either one of washing with water or an aqueous solution, or washing with a surfactant. Alternatively, the two may be used together. Furthermore, this washing may be combined with sonication.

【0037】

In the present invention, after selecting the plant embryo from the ground product, which was produced by grinding the plant seed as described above, the intact (capable of germinating) embryo produced by washing is minced, preferably in the presence of an extracting solvent whereafter the wheat embryo extract produced is separated and further purified, to produce a wheat embryo extract for cell-free protein synthesis.

In terms of the extracting solvent, aqueous solutions containing a buffer solution (for example, N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES)-KOH, piperazine-1, 4'-bis (2-ethanesulfonic acid) (PIPES)-NaOH, tris (hydroxymethyl) aminomethane (Tris)-HCl and the like, at a pH of 5 to 10), potassium ions, magnesium ions, and/or thiol reducing agents can be used. There are no particular restrictions on the thiol reducing agents but examples include DTT, 2-mercaptoethanol, glutathione/oxidized glutathione, thioredoxin, lipoic acid, cystein and the like. The concentrations for these reducing agents can be suitably selected according to the kind of reducing agent and can, for

example, be selected within the ranges of, for DTT, 10 μM to 5 mM, for 2-mercaptoethanol, 50 μM to 20 mM, and for glutathione/oxidized glutathione, 5 μM to 1 mM/1μM to 100 μM. Furthermore, calcium ions and L-amino acids and the like may be added as necessary. For example, solutions containing HEPES-KOH, potassium acetate, magnesium acetate, or L- amino acids or solutions wherein the method of Patterson *et al.* is partially modified (a solution containing HEPES-KOH, potassium acetate, magnesium acetate, calcium chloride, L-amino acids and/or DTT) can be used as the extracting solvent. The compositions and concentrations of the various components in the extracting solvent are already known *per se*, and compositions and concentrations commonly used in the preparation of wheat embryo extracts for cell-free protein synthesis may be adopted.

【0038】

The embryo is mixed with an amount of extracting solvent sufficient for extraction thereof and the embryo is minced in the presence of the extracting solvent. In terms of the amount of extracting solvent used for each gram of unwashed embryo, this is normally no less than 0.1 ml, preferably no less than 0.5 ml, and more preferably no less than 1 ml. There is no particular upper limit on the amount of extracting solvent, but this is normally no more than 10 ml, and preferably no more than 5 ml, for each gram of unwashed embryo. Furthermore, in terms of the embryo which is to be minced, this may be frozen as conventional, or an unfrozen embryo may be used, but the use of unfrozen embryo is preferred.

【0039】

In terms of the mincing method, conventional well-known methods, such as milling, crushing, impact or chopping may be used as the grinding method, but methods of mincing embryo by impact or chopping are preferred. Herein, the expression "mincing by impact or chopping" means breaking down the plant embryo under conditions that minimize, as compared to conventional milling or crushing, the breakdown of parts of the

plant embryo such as cellular membranes, cell walls, and organelles such as mitochondria, chloroplasts and the cell nucleus, and the like.

【0040】

There are no particular restrictions on the apparatus and methods that can be used in mincing the embryo, so long as the conditions described above are satisfied, but it is preferable that devices having a high-speed rotary blade, such as a Waring blender, be used. The speed of the rotating blade is normally no less than 1,000 rpm and preferably no less than 5,000 rpm, but is normally no greater than 30,000 rpm, and preferably no greater than 25,000 rpm. The running time for the rotating blade is normally no less than five seconds and preferably no less than 10 seconds. There is no particular upper limit on the running time, but this is normally no more than 10 minutes and preferably no more than five minutes. The temperature during mincing is preferably no greater than 10°C and is within a temperature range in which the mincing operation is possible. On the order of 4°C is particularly preferable.

【0041】

As a result of mincing the embryo by impact or chopping in this manner, the cell nucleus and cell walls of the embryo are not completely destroyed, but rather at least some portion thereof remains without having been broken down. That is to say, as the cellular membranes, cell walls and organelles such as the cell nucleus, and the like, of the embryo are not broken down to a greater degree than is necessary, it is possible to efficiently extract substances necessary to protein synthesis, such as RNA, ribosomes and the like, which are localized within the cytoplasm, at high degrees of purity, without contamination by impurities contained therein, such as and lipids and DNA.

【0042】

According to such a method, the conventional step of grinding the plant embryo and the conventional step of mixing the wheat embryo which has been grinded with an extracting solvent are carried out simultaneously, whereby wheat embryo

extract can be produced efficiently. The method described above is also referred to as the "blender method."

Next, the wheat embryo extract is recovered by centrifugation or the like and purified by gel filtration, dialysis or the like, allowing for the production of wheat embryo extract. Gel filtration may, for example, be performed using a Sephadex G-25 column or the like. The compositions and concentrations of the various components in the gel filtration solution are already known *per se*, and compositions and concentrations commonly used in the preparation of wheat embryo extracts for cell-free protein synthesis may be adopted. Here, if a cell extract for cell-free protein synthesis is extracted under the highly reducing conditions described above, it is preferable that this cell extract be passed through a gel filtration support that has been pre-equilibrated with a buffer solution that does not contain the reducing agent or which contains the reducing agent at a lower concentration. There are no particular restrictions on the composition of the buffer solution, but the use of a solution containing HEPES-KOH (pH 7.6), potassium acetate, magnesium acetate or L-amino acids is preferred, as this absorbs approximately 97% of the reducing agent contained in the extract solution. Specifically, if extraction was performed on wheat embryo using an extract solution containing 1 mM of DTT as the reducing agent, a wheat embryo extract containing approximately 30 μM of DTT, as the final concentration, can be obtained. However, as the activity of wheat embryo extract having a lower concentration of reducing agent is made markedly inferior as a result of frozen storage, it is preferable that the process of removing the reducing agent be performed immediately before this is used in the translation reaction.

【0043】

Following gel filtration or dialysis, the embryo extract may be contaminated with microorganisms, and in particular, with spores such as those of filamentous bacteria (mold). It is, therefore, preferable that these microorganisms be

eradicated. Particularly, the proliferation of microorganisms is sometimes observed in long-term (more than one day) cell-free protein synthesis reactions. It is, therefore, important to prevent this. There are no particular restrictions on the means for eradicating microorganisms, but the use of antimicrobial filters is preferred.

There are no particular restrictions on the pore size for the filter, so long as this is a size capable of eradicating microorganisms with which the cell extract may be contaminated, but 0.1 to 1 μm is normally suitable and 0.2 to 0.5 μm is preferred. It is of note that the spore size of *Bacillus subtilis*, which is of the small class, is 0.5 μm x 1 μm and therefore the use of a 0.20 μm filter (for example the Minisart™ by Sartorius) is effective in removing spores. When filtering, it is preferable that a filter having a large pore size be used first, whereafter a filter having a pore size capable of eliminating microorganisms by which the cell extract may be contaminated is used.

【0044】

The cell extract obtained in this manner is purified so as to substantially completely remove endosperm that comprises substances, contained or retained by the source cell itself, which inhibit protein synthesis function (substances that act on mRNA, tRNA, translation factor proteins, ribosomes and the like so as to inhibit the function thereof such as tritin, thionine, ribonuclease and the like). Herein, the expression "purified so as to substantially completely remove endosperm" refers to wheat embryo extracts from which endosperm components have been removed to an extent that ribosomes are substantially not deadenylated. Furthermore, the expression "to an extent that ribosomes are substantially not deadenylated" means that the ribosome deadenylation is less than 7%, and preferably 1% or less.

【0045】

Furthermore, such cell extract for cell-free protein synthesis contains low molecular weight substances that inhibit

protein synthesis (hereinafter these are also referred to as "low molecular weight synthesis inhibitors"). Therefore, it is preferable that these low molecular weight synthesis inhibitors be removed by fractionation from the constituent components of the cell extract, based on differences in molecular weight. It suffices that the molecular weight of the substances to be eliminated (low molecular weight synthesis inhibitors) be less than that of the factors contained within the cell extract that are necessary to protein synthesis. Specifically, examples of molecular weights include those which are no greater than 14,000 to 50,000 Daltons, and preferably no greater than 14,000 Daltons.

【0046】

Commonly used methods, which are well-known *per se*, can be used as the method for eliminating the low molecular weight synthesis inhibitors from the cell extract, and specific examples include methods based on dialysis by way of a dialysis membrane, gel filtration, ultrafiltration and the like. From among these, methods based on dialysis (dialyzing) are preferred for such reasons as the ease of supplying the substance to the internal dialysis solution.

Hereinafter, an example of the use of dialysis is described in detail.

【0047】

Examples of dialysis membranes which can be used for dialysis include those having molecular weight cutoff of 50,000 to 12,000 Daltons. Specifically, the use of a regenerated cellulose membrane having a molecular weight cutoff of 12,000 to 14,000 Daltons (Viskase Sales, Chicago) and the Spectra/Pore 6 (Spectrum Laboratories Inc., CA, USA) having a molecular weight cutoff of 50,000, is preferred. A suitable amount of the aforementioned cell extract is placed within such a dialysis membrane and dialysis is performed according to normal methods. It is preferable that the period of time for which dialysis is performed be on the order of 30 minutes to 24 hours.

【0048】

When the low molecular weight synthesis inhibitors are eliminated, in cases where insoluble matter forms in the cell extract, by means of inhibiting this (hereinafter also referred to as "stabilizing the cell extract"), it is possible to increase the protein synthesis activity of the final cell extract obtained (hereinafter also referred to as "processed cell extract"). Specific methods for stabilizing the cell extract include methods wherein the elimination of the low molecular weight inhibitors is performed in a solution containing at least high-energy phosphate compounds, such as ATP, GTP or the like. The use of ATP as the high-energy phosphate compound is preferred. Furthermore, it is preferable that this be performed in a solution containing ATP and GTP, and more preferable that this be performed in a solution containing ATP, GTP and the 20 types of amino acids.

【0049】

When the low molecular weight synthesis inhibitors are to be eliminated from a solution containing these components (hereinafter also referred to as "stabilizing components"), the stabilizing components may be added to the cell extract beforehand, and this may be supplied to the process for eliminating low molecular weight synthesis inhibitors after incubation. If dialysis is used for the elimination of low molecular weight synthesis inhibitors, the low molecular weight synthesis inhibitors can be eliminated by dialyzing with stabilizing components added not only to the cell extract, but also to the external dialysis solution. Adding a stabilizing component to the external dialysis solution is preferable as, even if the stabilizing component is broken down during dialysis, a new stabilizing component is continuously supplied. This can also be applied when gel filtration or ultrafiltration is used, and the same effect can be achieved by equilibrating the various supports with a filtration buffer solution containing a stabilizing component, whereafter a cell extract containing the stabilizing component is supplied and filtration is performed by adding more of the buffer solution.

【0050】

The amount of stabilizing component to be added and the time for the stabilization treatment may be suitably chosen according to the type of cell extract and the preparation method. Methods for selecting the same include those wherein various different amounts and types of stabilizing component are experimentally added to the cell extract and, after a suitable amount of time, the process for eliminating low molecular weight synthesis inhibitors is performed, whereafter the soluble fraction and the insoluble fraction are separated by such methods as centrifuging the processed cell extract produced, and the stabilizing component for which the least amount of insoluble matter was formed is chosen. Furthermore, a method is also preferred wherein the processed cell extracts obtained are used to perform cell-free protein synthesis, and a cell extract having high protein synthesis activity is chosen. Furthermore, the selection methods described above also include methods wherein, in cases where dialysis is used for the process of eliminating low molecular weight synthesis inhibitors, suitable stabilizing components are added also to the external dialysis solution and dialysis is performed for a suitable period of time using these, whereafter selection is made according to the amount of insoluble matter in the cell extract or the protein synthesis activity of the cell extract produced and the like.

【0051】

Specific examples of stabilization conditions for cell extracts selected in this manner include, in the case of eliminating low molecular weight synthesis inhibitors by way of dialysis with the wheat embryo extract prepared using the blender method, adding 100 μM to 0.5 mM of ATP, 25 μM to 1 mM of GTP and 25 μM to 5 mM of each of the 20 types of L-amino acid to the wheat embryo extract and the external dialysis and dialyzing for 30 minutes to 1 hour or more. If dialysis is used, this may be performed at any temperature, so long as it is a temperature that does not impair protein synthesis activity and

at which dialysis is possible. Specifically, the minimum temperature is a temperature at which the solution does not freeze, normally -10°C and preferably -5°C, and the maximum temperature is the limit for avoiding negative impact on the solution used for dialysis, which is 40°C and preferably 38°C.

【0052】

There are no particular restrictions on the method for adding the stabilizing component to the cell extract, but this may be added before the process for eliminating low molecular weight synthesis inhibitors, incubated for a suitable period of time so as to achieve stabilization, whereafter the process for eliminating low molecular weight synthesis inhibitors may be performed. Alternatively the process for eliminating low molecular weight synthesis inhibitors may performed using a cell extract to which the stabilizing component has been added and/or using a buffer solution to which this stabilizing component has been added for the purpose of use in this elimination process.

【0053】

Protein synthesis may be performed with the cell extract for cell-free protein synthesis described above by adjusting the concentration range of reducing agent to the range described above, adding the energy source, amino acids, translation template or tRNA and the like necessary for the cell-free protein synthesis, which is placed in a system or an apparatus well known *per se* that has been separately selected. Systems and apparatus for protein synthesis include the batch method (Pratt, J. M. *et al.*, *Transcription and Translation*, Hames, 179-209, B. D. & Higgins, S. J., eds., IRL Press, Oxford (1984)), or the continuous cell-free protein synthesis system, which continuously supplies the amino acids, the energy source and the like to the reaction system (Spirin, A. S., *et al.*, *Science*, 242, 1162-1164 (1988)), the dialysis method (Kikawa *et al.*, 21st Meeting of The Molecular Biology Society of Japan, WID6), or the overlay method (Sawasaki, T., et al., FEBS Let., 514, 102-105(2002)).

【0054】

Furthermore, such methods may be used as those wherein the template RNA, the amino acids, the energy source and the like are supplied to the synthesis reaction system when necessary, and the synthesis products and breakdown products are removed when necessary (JP-2000-333673-A, hereinafter also referred to as "discontinuous gel filtration").

From among these methods, the use of systems that continuously or discontinuously supply amino acids and an energy source allows the reaction to be maintained over a long period of time, which makes further increases in efficiency possible, but when performing protein synthesis using the weakly reducing synthesis reaction solution of the present invention, the batch method is preferred, as this tends to improve protein synthesis efficiency. Furthermore, if a wheat embryo extract is prepared by the blender method described above, it normally contains a sufficient amount of tRNA, so that it is not necessary to add tRNA.

【0055】

When protein synthesis is performed by way of the batch method, for example, the synthesis reaction solution without a translation template, is pre-incubated for a suitable period of time as necessary, whereafter the translation template is added and protein synthesis is performed by incubation, and the like. In terms of the reaction solution, this may for example contain 10 to 50 mM of HEPES-KOH (pH 7.8), 55 to 120 mM of potassium acetate, 1 to 5 mM of magnesium acetate, 0.1 to 0.6 mM of spermidine, 0.025 to 1 mM of each of the L-amino acids, 20 to 70 μM, preferably 30 to 50 μM of DTT, 1 to 1.5 mM of ATP, 0.2 to 0.5 mM of GTP, 10 to 20 mM of creatine phosphate, 0.5 to 1.0 U/μl of RNase inhibitor, 0.01 to 10 μM of protein disulfide isomerase and 24 to 75% of wheat embryo extract (prepared by the blender method).

【0056】

When such a translation reaction solution is used, the pre-incubation is at 10 to 40°C for 5 to 10 minutes and the

incubation is likewise at 10 to 40°C, preferably 18 to 30°C, and more preferably 20 to 26°C. The reaction time is the time until the reaction stops, and in the batch method this is normally on the order of 10 minutes to 7 hours.

If protein synthesis is performed by means of the dialysis method, the synthesis reaction solution is made the internal dialysis solution and a device is used whereby this is separated from the external dialysis solution by a dialysis membrane, through which substances can transport, whereby protein synthesis is performed. Specific examples include those wherein the synthesis reaction solution described above, without a translation template, is pre-incubated for a suitable period of time as necessary, whereafter the translation template is added, whereafter this is placed in a suitable dialysis chamber as the internal reaction solution. Examples of dialysis containers include containers with a dialysis membrane at the bottom (Daiichi Kagaku; Dialysis Cup 12,000 or the like), or a dialysis tube (Sanko Junyaku: 12,000 or the like). The dialysis membrane used may have a molecular weight cutoff of 10,000 Daltons or more, those with a molecular weight cutoff on the order of 12,000 Daltons being preferred.

【0057】

The aforementioned synthesis reaction solution, without the translation template, is used as the external dialysis solution. It is possible to improve dialysis efficiency by replacing the external dialysis solution with fresh dialysis solution when the reaction speed drops. The reaction temperature and time are suitably selected according to the protein synthesis system to be used, but in systems wherein wheat embryo extract is used, this is normally performed at 10 to 40°C, preferably 18 to 30°C, and more preferably 20 to 26°C, for 10 minutes to 12 days.

【0058】

When the protein synthesis is carried out using the overlay method, the synthesis reaction solution is placed in a suitable container, and the external dialysis solution

described above in the dialysis method is overlaid on top of this solution(the synthesis reaction solution) so as not to disturb the interface, so as to carry out the protein synthesis. Specific examples include those wherein the synthesis reaction solution described above, without a translation template, is pre-incubated for a suitable period of time as necessary, whereafter the translation template is added, whereafter this is placed in a suitable container as the reaction phase. Examples of the container include a microtiter plate or the like. The external dialysis solution described above in the dialysis method (supply phase) is overlaid on the top layer of this reaction phase so as not to disturb the interface, and the reaction is performed.

【0059】

In addition, the interface between the two phases does not have to be formed by superposition in a horizontal plane; a horizontal plane can also be formed by centrifuging a mixture that contains both phases. When the diameter of the circular interface between the two phases is 7 mm, a volume ratio of the reaction phase and the supply phase of 1:4 to 1:8 is suitable, and 1:5 is preferred. The rate of exchange of substances due to diffusion increases with the area of the interface formed by the two phases, increasing the protein synthesis efficiency. Therefore, the volume ratio of the two phases changes according to the area of the interface between the two phases. The synthesis reaction is carried out under static conditions, and the reaction temperature and time are suitably selected for the protein synthesis system to be used, but in systems using wheat embryo extract, this can be performed at 10 to 40°C, preferably 18 to 30°C and more preferably at 20 to 26°C, normally for 10 to 17 hours. Furthermore, when *E. coli* extract is used, a reaction temperature of 30 to 37°C is suitable.

【0060】

When the protein synthesis is carried out using the discontinuous gel filtration method, the synthesis reaction is performed by way of the synthesis reaction solution, and when

the synthesis reaction stops, the template RNA, the amino acids, the energy source and the like are supplied, and the products of synthesis or degradation are evacuated, so as to perform protein synthesis. Specific examples include those wherein the synthesis reaction solution described above, without a translation template, is pre-incubated for a suitable period of time as necessary, whereafter the translation template is added, this is placed in a suitable dialysis chamber and the reaction is performed. Examples of the container include a micro plate or the like. In this reaction, when, for example, the reaction solution contains 48% by volume of wheat embryo extract, the synthesis reaction stops completely in 1 hour. This can be verified by measuring the incorporation of amino acids into the protein or by an analysis of polyribosomes by centrifugation over a sucrose density gradient (*Proc. Nat'l. Acad. Sci.* USA, 97, pp. 559-564 (2000)). The synthesis reaction solution in which the synthesis reaction has stopped is passed through a gel filtration column, which has been pre-equilibrated with a supply solution that has the same composition as the external dialysis solution described in the dialysis method. The synthesis reaction is resumed by re-incubating the filtered solution at a suitable reaction temperature, and the protein synthesis proceeds over several hours. Thereafter, these reaction and gel filtration operations are repeated. The reaction temperature and time are suitably selected according to the protein synthesis system to be used, but in systems wherein wheat embryo extract is used, gel filtration is preferably repeated once every hour at 26°C.

【0061】

When bonding a labeling substance to the single chain antibody of this invention in the presence of a specific enzyme according to this kind of cell-free protein translation, the above-described translation reaction is conducted in the presence of the labeling substance and an enzyme that is capable of binding the labeling substance to a polypeptide of a linker part. More specifically, when bonding biotin as a labeling

substance to a linker, a translation reaction is conducted in the presence of, for example, a biotin ligase (Avidity, manufactured by LLC, or the like) that is an enzyme that bonds biotin by recognizing an amino acid recognized by a biotin ligase that is previously inserted into a linker. An added amount of biotin and the biotin ligase is preferably an amount described in the instructions accompanying a commercially available product (enzyme).

【0062】

When bonding labeling substances after synthesis of the protein, after completing the translation reaction bonding may be conducted to a linker part of the single chain antibody in the translation reaction solution by a method suitable for the respective labeling substances, or bonding may be conducted by a method suitable for the respective labeling substances after purifying the single chain antibody by the method described below.

The thus obtained single chain antibody or labeled single chain antibody of this invention can be confirmed by a known method. More specifically, for example, a method involving measuring incorporation of amino acid into protein, separation by SDS-polyacrylamide gel electrophoresis and staining by Coomassie brilliant blue (CBB), or autoradiography (Endo, Y., et al., J. Biotech., 25, 221-230 (1992); Proc. Natl. Acad. Sci. USA, 97, 559-564 (2000)) or the like can be used.

【0063】

Further, since the single chain antibody or labeled single chain antibody of interest is contained at a high concentration in the thus-obtained reaction solution, the single chain antibody or labeled single chain antibody of interest can be easily acquired from the reaction solution by a known method of separation and purification, such as dialysis, ion-exchange chromatography, affinity chromatography or gel filtration.

【0064】

(3) Utilization of labeled single chain antibody

The labeled single chain antibody of this invention can be used in a method for analyzing an antigen-antibody reaction by analyzing the binding ability thereof against an antigen. Specifically, in case a labeled material is an immobilization material, the antibody is immboilized to analyze affinity against antigen thereby obtaining result of antigen-antibody reaction. Also, in case a labeled material is signal material, the antibody is immboilized to analyze affinity against antigen thereby obtaining result of antigen-antibody reaction.

【0065】

As method of preparing an immboilizied single antibody, an adaptor material is immobilized in suitable plate and the immbolized single antibody is contacted with the palate to remove nonbonding antibody. As a reaction plate used in the aforementioned antigen-antibody analysis method, a reaction plate that is suitable for an apparatus or method for analyzing an antigen-antibody reaction can be used. More specifically, when conducting analysis by enzyme-linked immunosorbent assay (ELISA) (Crowthjer, J. R., Methods in Molecular Biology, 42, (1995)), a plastic microtiter plate that is normally used in the ELISA method is preferred. When using a surface plasmon resonance method (Cullen, D. C., et al., Biosciences, 3(4), 211-225 (1987-88)), a reaction plate in which a metallic thin film of gold, silver, platinum or the like is formed on a transparent reaction plate made of glass or the like is preferred. Further, when using molecule imaging using an evanescent field (Funatsu, T., et al., Nature, 374, 555-559 (1995)), a transparent medium made of glass or the like is preferable, and more preferably a reaction plate made of quartz glass is used. When using fluorescent imaging analysis, a nylon membrane or nitrocellulose membrane that is normally used for immobilizing a protein or the like can be used, and a plastic microtiter plate or the like can also be used. Further, a complex carbohydrate (for example, agarose and sepharose), acrylic resin (for example, polyacrylamide and latex beads), magnetic beads, silicon wafer and the like can also be used as

reaction plates.

【0066】

Bonding of an adapter substance to this kind of reaction plate can be performed according to a known method that is conventionally used in the art. More specifically, a diazo process, a peptide process (using acid amide derivatives, carboxychloride resin, maleic anhydride derivatives, isocyanate derivatives, cyanogen bromide activated polysaccharides, cellulose carbonate derivatives or the like), alkylation process, a method using a crosslinking reagent, a method using Ugi reaction and the like may be mentioned. When using a reaction plate made of glass or the like, a method that conducts physical adsorption can also be used. Further, a commercially available product such as streptavidin magnetic beads (manufactured by Promega Corp.) can also be used.

【0067】

By bringing the thus obtained labeled single chain antibody into contact with a solution containing one or more test substances such as known antigens and analyzing the antigen-antibody reaction, it is possible to identify an antibody having binding specificity with respect to the antigen. The antigen may be a protein, or may be an organic compound, carbohydrate, nucleic acid or the like. These may be isolated, or may be recombinant or naturally occurring substances. The amount of an antigen used herein is preferably in the range of approximately 1 to 100 ng/μl. The time required for an antigen-antibody reaction is normally within the range of 5 minutes to 24 hours, and in general a time between 0.5 to 2 hours is preferable.

【0068】

When a single chain antibody was immobilized, a method that is known in the art can be used to reduce nonspecific adsorption. Specific examples thereof include a method of precoating an array solid support using bovine serum albumin (BSA), reduced low fat milk, salmon sperm DNA, pig (mucosal) heparin or the like (Ausubel et al., Short Protocols in

Molecular Biology, 3rd edition (1995)).

【0069】

After the antigen-antibody reaction, in the case of an immobilized single chain antibody, a step can be added of washing the solid phase to which the antibody is bound using a buffer containing surfactants or the like that can be used biochemically. The composition of the buffer and the number of washings and the like can be appropriately selected in accordance with the strength of the antigen-antibody reaction and the like.

A method for quantitatively or qualitatively determining interaction between a labeled single chain antibody and a test substance can be conducted according to a known method that is conventionally used in the art. More specifically, a method such as ELISA, surface plasmon resonance, molecular imaging utilizing an evanescent field, fluorescent imaging analysis or a method using radioisotope labels may be mentioned.

【0070】

A test substance such as an antigen may be any substance that may comprise an antigen. Specific examples thereof include body fluid such as blood, bacterial cell wall extract, and a protein mixture.

According to the method for analyzing an antigen-antibody reaction using the labeled single chain antibody of this invention and a reagent kit for measuring an antigen-antibody reaction comprising a reagent used in the analysis method, for example, a tool for diagnosing and analyzing the presence or absence of a human autoantibody, a cancer cell specific antigen and the like can be provided.

【0071】

【Examples】

This invention is described in further detail hereunder by means of examples, however the scope of this invention is not limited by these examples.

Example 1. Preparation of biotinylated anti-Salmonella single chain antibody

(1) Preparation of DNA encoding Salmonella single chain antibody and linker

An anti-Salmonella single chain antibody was selected as the single chain antibody of this invention to conduct the following test. The X-ray conformation of this antibody has already been analyzed, and molecular recognition with respect to sugar chain has been investigated in detail (Cygler, M., et al., Science, 253, 442-445 (1991); Bundle, D. R., et al., Biochemistry, 33, 5172-5182 (1994)). Lipopolysaccharide is present on the cell cortex of Salmonella bacteria, and anti-Salmonella antibody binds to O-antigen that is located at the most extracellular domain of the lipopolysaccharide (Anand, N. N., et al., Protein Engin., 3, 541-546 (1990)). It has been reported that a single chain antibody in which VL chain and VH chain, antigen-recognition sites that bind specifically to O-antigen, were connected by a specific linker was expressed in large quantities using *Escherichia coli* (Anand, N. N., et al., J. Biol. Chem., 266, 21874-21879 (1991)). Since a formation in which one disulfide bond is present respectively in the VL chain and VH chain is indispensable to synthesize a single chain antibody in an active form (Zdanov, A. L. Y., et al., Proc. Natl. Acad. Sci. USA, 91, 6423-6427 (1994)), the aforementioned single chain antibody was used as the subject of the method of this invention.

【0072】

DNA encoding anti-Salmonella single chain antibody was acquired by conducting a polymerase chain reaction (PCR) employing a plasmid containing DNA encoding a single chain antibody against wild-type Salmonella O-antigen (Anand, N. N., et al., J. Biol. Chem., 266, 21874-21879 (1991)) as a template and using primers comprising the nucleotide sequences represented by SEQ ID NOS: 2 and 3. The acquired DNA fragments were ligated into pGEMT-easy Vector (from Promega Corp.), and then digested with the restriction enzymes BgIII and NotI. The obtained DNA fragments were inserted into pEU vector that had been previously digested with the same restriction enzymes.

PCR was conducted employing this plasmid as a template and using primers comprising the nucleotide sequences represented by SEQ ID NOS: 4 and 5 to introduce a stop codon. The thus produced plasmid was designated "scfv-pEU".

【0073】

Next, DNA was produced in which a DNA sequence (SEQ ID NO: 1) encoding a biotin ligase recognition sequence was inserted in a linker part. First, PCR was carried out employing as a template the plasmid scfv-pEU produced as described above, and using LA Taq (manufactured by Takara Co., Ltd.) kit with primers comprising the nucleotide sequences represented by SEQ ID NOS: 6 and 7. The PCR reaction solution was prepared using 5 µl of 10x LA buffer, 5 µl of 25 mM magnesium chloride, 8 µl of 2.5 mM dNTP, 1 µl of 20 µM primer (for each primer), and 0.1 ng of template plasmid/50 µl, and reaction was conducted at 94 °C for 1 min × 1 cycle, 94 °C for 45 sec/ 55 °C for 1 min/ 72 °C for 1.5 min × 30 cycles, and then 72 °C for 5 min. In accordance with a conventional method, the ends of amplified DNA fragments were blunted using KOD T4 polymerase (manufactured by NEB Inc.), the fragments were phosphorylated with Polynucleotide Kinase (NEB Inc.), and self-ligation was then carried out using Ligation High (manufactured by Toyobo Co., Ltd.) to produce a circular plasmid (FIG. 1; hereafter, this is sometimes referred to as "scFv-biotin-pEU").

【0074】

(2) Preparation of cell extract for weak reduced form of cell-free protein synthesis

Hokkaido-produced Chihoku wheat seeds (unsterilized) were added to a mill (Rotor Speed mill pulverisette 14 model, manufactured by Fritsch Inc.) at a rate of 100 g per minute, to gently pulverize the seeds at a rotation speed of 8,000 rpm. After sieving to collect fractions containing embryo having germinating capacity (mesh size 0.7 to 1.00 mm), a floating fraction containing embryo with germinating capacity was collected by flotation using a mixed solution of carbon tetrachloride and cyclohexane (volume ratio = carbon

tetrachloride: cyclohexane = 2.4: 1), the organic solvent was removed by drying at room temperature, and impurities such as seed coat that were mixed therein were removed by blowing air at room temperature to obtain a coarse embryo fraction.

【0075】

Next, embryo was selected from the coarse embryo fraction by utilizing difference in color using a belt type color sorter BLM-300K (manufactured by Anzai Manufacturing Co. Ltd.; selling agent: Anzai Corporation, Ltd.) in the manner described below. The color sorter is an apparatus having means to irradiate light on a coarse embryo fraction, means to detect reflected light and/or transmitted light from the coarse embryo fraction, means to compare detected values and reference values, and means to select and remove components with a detected value that is outside the reference values or components with a detected value that is within the range of reference values.

【0076】

Coarse embryo fractions were supplied onto a beige color belt of the color sorter to form an amount of 1000 to 5000 grains/cm^2, light was irradiated by fluorescent lamp onto the coarse embryo fractions on the belt, and the reflected light was detected. The conveying speed of the belt was 50 m/min. A monochrome CCD line sensor (2048 pixels) was used as a light-receiving sensor.

First, in order to remove black-colored components (seed coat etc.) from the embryo, the reference value was set between the brightness of the embryo and the brightness of the seed coat, and components having a value outside the reference value were removed by suction. Subsequently, in order to screen for endosperm, the reference value was set between the brightness of the embryo and the brightness of endosperm, and components having a value outside the reference value were removed by suction. Suction was conducted using 30 suction nozzles provided at positions of about 1 cm apart on the upper part of the conveyor belt (the suction nozzles were arranged in a condition of 1 nozzle per 1 cm length).

【0077】

By repeating this method, embryo was screened until the purity of the embryo (weight ratio of embryo contained per 1 g of arbitrary sample) was 98% or more.

The obtained wheat embryo fraction was suspended in distilled water with a temperature of 4 °C, and washed using an ultrasonic washer until the cleaning fluid lost its white turbidity. Next, the fraction was suspended in a solution containing 0.5 % Nonidet P40 (manufactured by Nacalai Tesque Inc.), and washed using an ultrasonic washer until the cleaning fluid lost its white turbidity to obtain wheat embryo, after which the following process was conducted at 4 °C.

【0078】

Extracting solvent (80 mM HEPES-KOH (pH 7.8), 200 mM potassium acetate, 10 mM magnesium acetate, and 8 mM dithiothreitol (0.6 mM each of 20 kinds of L-form amino acid may also be added)) of two-fold volume relative to the wet weight of the washed embryo was added thereto, and limited pulverization of the embryo was conducted 3 times for 30 seconds each time at 5,000 to 20,000 rpm using a Waring blender. Centrifuged supernatant obtained from this homogenate by centrifugation at 30,000× g for 30 min using a high-speed centrifuge was centrifuged again under the same conditions to obtain supernatant. A decline in activity was not observed for this sample after long-term storage at -80 °C or below. The obtained supernatant was filtrated through a filter with a pore size of 0.2 μM (New SteraDisk 25; manufactured by Kurabo Industries Ltd.) for filter sterilization and removal of minute contaminants.

【0079】

Next, this filtrate was subjected to gel filtration using a Sephadex G-25 column that was previously equilibrated with a mixed solution (40 mM HEPES-KOH (pH 7.8), 100 mM potassium acetate, 5 mM magnesium acetate, and 0.3 mM each of 20 kinds of L-form amino acids (the amino acids may be omitted in accordance with the purpose of protein synthesis, or labeled

amino acids may be used). After centrifuging the obtained filtrate again at 30,000× g for 30 min and adjusting the concentration of the collected supernatant so that A260 nm was 90 to 150 (A260/A280 = 1.4 to 1.6), the supernatant was stored at -80 °C or below until use in the protein synthesis reaction or dialysis process described hereunder.

【0080】

(3) Protein synthesis using weak reductive translation reaction solution (when adding biotin and biotin ligase at time of translation)

Transcription was conducted for the translation template DNA acquired in the above (1) using SP6 RNA polymerase (manufactured by Toyobo Co., Ltd.). The reaction solution contained 80 mM HEPES-KOH (pH 7.6), 16 mM magnesium acetate, 2 mM spermidine, 10 mM DTT, NTPs (2.5 mM each), 0.8 U/μl RNase inhibitor, 50 μg/ml plasmid, and 1.2 U/μl SP6 RNA polymerase/ddw 400 μl. After incubation for 2 hours at 37 °C, purification was conducted by phenol/chloroform extraction and passage over a Nick column (manufactured by Amersham Pharmacia Inc.), followed by ethanol precipitation, and the ethanol precipitate was dissolved in 35 μl of purified water.

【0081】

Translation reaction was conducted using the obtained mRNA. The translation reaction solution consisted of 1.2 mM ATP, 0.25 mM GTP, 15 mM creatine phosphate, 0.4 mM spermidine, 29 mM HEPES-KOH (pH 7.6), 95 mM potassium acetate, 2.7 mM magnesium acetate, 0.23 mM L-form amino acids, 0.58 U/μl RNase inhibitor (manufactured by Promega Corp.), 4nCi/μl 14C-Leu, 7.5 μg mRNA, 0.5 μM PDI, 19.5 μM biotin (Nacalai Tesque Inc.), 19.5 μg/μl biotin ligase (manufactured by Avidity Inc.), and 12 μl wheat embryo extract. Reaction was conducted by batch method for 3 hours at 26 °C. A translation reaction to which biotin was not added was also conducted as a control.

【0082】

The reaction solution after 3 hours of translation reaction was centrifuged for 10 minutes at 15,000 rpm to

separate solubilized components, and unreacted biotin remaining therein was removed using a G-25 spin column that was equilibrated with 50 mM Tris (pH 8.0). After diluting 20 µl of the spin column eluate with an equivalent amount of 50 mM Tris (pH 8.0) buffer solution, 5 µl of streptavidin magnetic beads (manufactured by Promega Corp.) was added, and this was mixed gently at room temperature. After collecting the magnetic beads using a magnetic field, supernatant fractions were acquired, and after separation by SDS-PAGE, the amount of anti-Salmonella single chain antibody was determined by autoradiography. The result is shown in co-transl. biotinylation of FIG. 2. As can be seen from the figure, when translation was conducted in the presence of biotin and a biotin ligase (in the figure: +biotin), the amount of antibody collected by magnetic beads through bonding with streptavidin was large, and conversely, when biotin was not added to the reaction solution (-biotin), almost no antibodies bound to the magnetic beads. It was thus clarified that biotin binds to anti-Salmonella single chain antibody according to the above-described method.

【0083】

(4) Protein synthesis using weak reductive translation reaction solution (when adding biotin and biotin ligase after translation reaction)

Biotin and a biotin ligase were added at 3 hours passage after the start of translation reaction, in a similar manner to the method of anti-Salmonella single chain antibody described in the above (1) to (3). The results are shown in post-transl. biotinylation of FIG. 2. As can be seen from the figure, it was found that for both the reaction solution to which biotin was added (+) and the reaction solution to which biotin was not added (-), almost no biotinylated antibody was removed with the magnetic beads. It was thus found that biotin ligase and biotin are preferably added during the translation reaction.

【0084】

Example 2 Immobilization of biotinylated single chain antibody and analysis of antigen-antibody reaction

(1) Preparation of aldehyded Salmonella O-antigen

20 mg (2.8 μmol) of lipopolysaccharide (manufactured by Sigma Chemical Co., Ltd.) was dissolved in 20 μL of 0.25 M sodium hydroxide aqueous solution and stirred for 1 hour at 56 °C. After dialysis against distilled water, 200 mg (0.8 mmol) of sodium metaperfolate was added, and this mixture was stirred for 5 minutes while shading from light. After further adding 1 ml of ethylene glycol and stirring for 1 hour, the resulting mixture was subjected to dialysis against distilled water, and the dialysis residue was lyophilized to obtain powder of aldehyde-type Salmonella sugar chain. This powder was dissolved in 0.2 ml of 20 mM sodium borate buffer (pH 9.0) (10 mg/ml). After 3 times washing 0.1 ml of aminated magnetic beads (NH2-Mag; manufactured by Polyscience Inc.) with 0.4 ml of the same buffer to equilibrate, the beads were added to the above aldehyde-type Salmonella sugar chain solution, and reaction was conducted for 6 hours at room temperature. The magnetic beads were then washed 3 times with 0.4 ml of the same buffer. The ratio of immobilization of sugar chain onto the magnetic beads was obtained by determining the quantity of sugar chain remained in the supernatant by a phenol/sulfuric acid process. The binding ratio of sugar chain to the magnetic beads was 40% (0.13 μmol Salmonella sugar chain/ 100 μl magnetic beads).

【0085】

(2) Bonding of biotinylated anti-Salmonella single chain antibody and Salmonella sugar chain

After synthesizing biotinylated anti-Salmonella single chain antibody by the method described in Example 1 (total 38 μl), excess biotin was removed by gel filtration with a G-25 spin column that was equilibrated with 10 mM PBST (pH 8.0) and 0.6 mM $CaCl_2$. 40 μl of the protein solution was added onto a 96-well microplate together with 10 μl of immobilized Salmonella antigen (Sal-Mag) produced in the above (1) that had been previously washed with 25 μl of wheat embryo extract

containing 0.6 mM $CaCl_2$. After gently mixing for 15 minutes, washing was performed 4 times with 40 µl of 0.15 M NaCl/10 mM PBST (pH 8.0), and finally elution was conducted 4 times using an equivalent amount of 0.1 M glycine-HCL (pH 2.4). Single chain antibody that had not bound to the antigen was eluted by the initial washing, and single chain antibody that had bound to the antigen was eluted by the elution conducted thereafter. The amount of protein in each fraction (5 µl) was determined by 14 C count. The result is shown in FIG. 3. Here, for the purpose of confirming that the biotinylated single chain antibody retaine antigen specificity, the binding result for a case of biotinylating mutant G102D in a similar manner is also shown. As can be seen from the figure, while for the wild type close to 50 % of the total antibody was present in an active fraction (no. 6) eluted with a pH acidic solution, in contrast, for the mutant G102D an active fraction was completely non-existent and most of the antibody appeared in the pass-through fraction no. 1. This result shows that the biotinylated anti-Salmonella single chain antibody retained its original antigen binding activity, and that co-translational biotinylation progresses without any loss of antigen binding activity.

【0086】

(3) Measurement of dissociation equilibrium constant by Biomolecular Interaction Analysis System (Iasys)

First, streptavidin (0.1 mg/ml; manufactured by Nacalai Tesque Inc.) was immobilized in a biotin cuvette (manufactured by Affinity Sensors Inc.) (immobilized amount: 674 arc sec., 27.2 ng, 0.97 pMol). Next, biotinylated single chain antibody prepared in Example 1 was purified using immobilized Salmonella sugar chain antigen (Sal-Mag) according to the method described in the above (2). 8.4 pmol/50 µl of purified biotinylated single chain antibody was obtained by being converted from a 14 C dpm value. This 50 µl amount was added to the above cuvette, and immobilized on the streptavidin (immobilized amount: 433.6 arc sec., 11.5 ng, 0.4 pmol). By adding thereto various

concentrations (2.4, 4.8, 9.7, 12.9, 19.4 μM) of Salmonella sugar chain, association and dissociation curves were determined. FIG. 4 shows the results, while Table 1 lists the dissociation equilibrium constant obtained from the curves. Table 1 also lists values obtained for single chain antibody synthesized using viable cells of *Escherichia coli* by the same method for comparison (MacKenzie, C. R. et al., J. Biol. Chem., 271, 1527-1533 (1996)). As can be seen from the response curve of FIG. 4, it was possible to immobilize biotinylated single chain antibody onto streptavidin, and furthermore, a function for binding an antigen was retained. As shown in Table 1, when the dissociation equilibrium constant Kd was calculated on the basis of this curve, it was found that the constant was in the order of 1×10^{-7}. Based on this result it was clarified that the single chain antibody prepared in Example 1 and immobilized by binding between biotin and streptavidin has a Kd value equivalent to that of complete anti-Salmonella antibody IgG.

【0087】

Table 1

antibody	K_D (M)	K_{diss} (S^{-1})	K_{ass} ($M^{-1}S^{-1}$) s
cell-free system	5.1×10	0.8×10	4.4×10
in-vivo system	6.5×10	3.1×10	1.8×10
IgG	1.4×10	1.2×10	8.7×10

【0088】

Comparative Example 1. Investigation of immobilization

efficiency according to biotin binding position

Addition of biotin to single chain antibody by chemical bonding

The method used in this example was in accordance with antibody labeling methods described in Immunobiochemical Methods, Biochemical Experiment Course, (Japanese Biochemical Society, Tokyo Kagaku Dojin (1986)).

By the method described in Example 1, a reaction solution was synthesized without adding biotin ligase and biotin at the time of translation reaction, and the solution was centrifuged at 15,000 rpm for 10 minutes to obtain supernatant. After diluting a 25 µl soluble fraction of supernatant with an equivalent amount of 1 M sodium bicarbonate solution, the buffer was exchanged using a G-25 Sephadex column, and 1 µl of NHS-biotin (N-hydroxysuccimide-biotin, 50 mg/ml DMSO) was then added. After reacting this solution over night at 4 °C, binding ability with an antigen was analyzed as described below.

【0089】

Analysis of binding activity with antigen

30 µl of reaction solution prepared in the above (1) was subjected to gel filtration with a G-25 spin column that was equilibrated with 10 mM of PBST (pH 8.0) and 0.6 mM of $CaCl_2$ to remove excess biotin. 40 µl of the protein solution was added onto a 96-well microplate together with 10 µl of immobilized Salmonella antigen (Sal-Mag) produced in Example 2 (1) that had been previously washed with 25 µl of wheat embryo extract containing 0.6 mM of $CaCl_2$. After gently mixing for 15 minutes, washing was conducted 4 times with 40 µl of 0.15 M NaCl/10 mM PBST (pH 8.0), and finally elution was conducted 4 times using an equivalent amount of 0.1 M glycine-HCL (pH 2.4). FIG. 5 shows the result. If the antibody retained its activity it would be eluted by the latter acidic buffer, however, as can be seen from the figure, the presence of protein was not observed in fraction numbers 10 to 13, and most of the protein was present in the first pass-through fraction. This result shows that the biotinylated single chain antibody produced by the aforementioned chemical process lost its antigen-binding

activity.

【0090】

Example 3. Production of single chain antibody inserted with polyhistidine peptide and immobilization thereof

(1) Production of single chain antibody containing polyhistidine peptide in a linker part

PCR was conducted employing scfv-pEU described in Example 1 (1) as a template, and using LA Taq (manufactured by Takara Co., Ltd.) kit with primers comprising the nucleotide sequences represented by SEQ ID NOS: 8 and 9. The PCR reaction solution was prepared with 5 µl of 10× LA buffer, 5 µl of 25 mM magnesium chloride, 8 µl of 2.5 mM dNTP, 1 µl of 20 µM primer (for each primer), and 0.1 ng of template plasmid/50 µl. The reaction was conducted by heating at 94 °C for 1 min × 1 cycle, 94 °C for 45 sec/ 55 °C for 1 min/ 72 °C for 1.5 min × 30 cycles, and then 72 °C for 5 min. In accordance with a conventional method, the ends of amplified DNA fragments were blunted using KOD T4 polymerase (manufactured by NEB Inc.), the fragments were phosphorylated with Polynucleotide Kinase (NEB Co., Ltd.), and self-ligation was then carried out using Ligation High (manufactured by Toyobo Co., Ltd.) to produce a circular plasmid (FIG. 1; hereafter, this is sometimes referred to as "scFv-pHIS-pEU").

【0091】

After conducting transcription according to the method described in Example 1 (3) employing this plasmid as a template and purifying the mRNA, DTT in the translation reaction solution was replaced with 200 µM of mercaptoethanol to conduct a translation reaction. The reaction solution after 3 hours of translation reaction was centrifuged for 10 minutes at 15,000 rpm to separate solubilized components, and excess mercaptoethanol was removed using a G-25 spin column that was equilibrated with 50 mM phosphate buffer (pH 7.0), 500 mM NaCl, and 5% glycerol (binding buffer).

【0092】

After diluting 20 µl of the spin column eluate with an

equivalent amount of the binding buffer, 80 µl of the solution was passed over a 200 µl nickel column (50% resin) (metal affinity resin, Talon) that was previously washed 6 times with 150 µl of binding buffer, and this was incubated for one hour at room temperature. This column was washed 4 times (w1 to w4 in the figure) with 150 µl of 50 mM phosphate buffer (pH 7.0), 500 mM NaCl, 5% glycerol, and 6 mM imidazole (washing buffer), and elution was then carried out 5 times (e1 to e5 in the figure) with 150 µl of 50 mM phosphate buffer (pH 7.0), 500 mM NaCl, and 150 mM imidazole (elution buffer). The amount of single chain antibody contained in each fraction was measured by ^{14}C dpm value. FIG. 6 shows the result. In the figure, C indicates the ^{14}C dpm value in the total amount of protein-containing solution prior to passage over the column. The horizontal axis of the graph shows fraction numbers, w1 to w4 show the ^{14}C dpm values in fractions eluted by the washing buffer, while e1 to e5 show the ^{14}C dpm values in fractions eluted by the elution buffer. ET shows the total of the ^{14}C dpm values for e1 to e5.

【0093】

Fraction numbers e1 to e5 indicate the existence of single chain antibody containing polyhistidine peptide binding specifically with nickel. As can be seen from the figure, it was found that approximately 50% of the total synthesized amount of single chain antibody could be purified by nickel column. It was confirmed by the method described in Example 2 that the purified single chain antibody also retained antigen-binding activity. This result indicates that a single chain antibody having polyhistidine peptide incorporated in a linker part thereof can be immobilized to a nickel solid phase in a condition in which it retains its activity.

【0094】

【Effect of invention】

According to the present invention, there is provided a single chain antibody that retains activity for binding specifically with an antigen. The single chain antibody can

be bound to a solid phase via a labeling substance, and can be used to produce an antibody chip or the like. By synthesizing this single chain antibody using a cell-free protein translation system that allows an intramolecular disulfide bond to be retained, there can be provided an antibody having specific binding ability against an antigen that is higher than that of an antibody synthesized within a viable cell such as *Escherichia coli*.

【0095】

【Sequence list】

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【0096】

【BRIEF DESCRIPTION OF THE DRAWINGS】

FIG. 1 is a view showing the structure of a translation template of the single chain antibody of this invention.

FIG. 2 is a photograph of electrophoresis showing the degree of binding of biotin to a single chain antibody caused by a biotin ligase.

FIG. 3 is a view showing the degree of specific binding to an antigen of the labeled single chain antibody of this invention

FIG. 4 is a view showing a curve of association and dissociation of the labeled single chain antibody of this invention and an antigen.

FIG. 5 is a view showing the degree of binding between an antigen and a single chain antibody in which biotin was bonded in an area other than a linker part.

FIG. 6 is a view showing the degree of binding to a nickel column of a single chain antibody having a polyhistidine peptide in a linker part.

Drawings

Fig.1

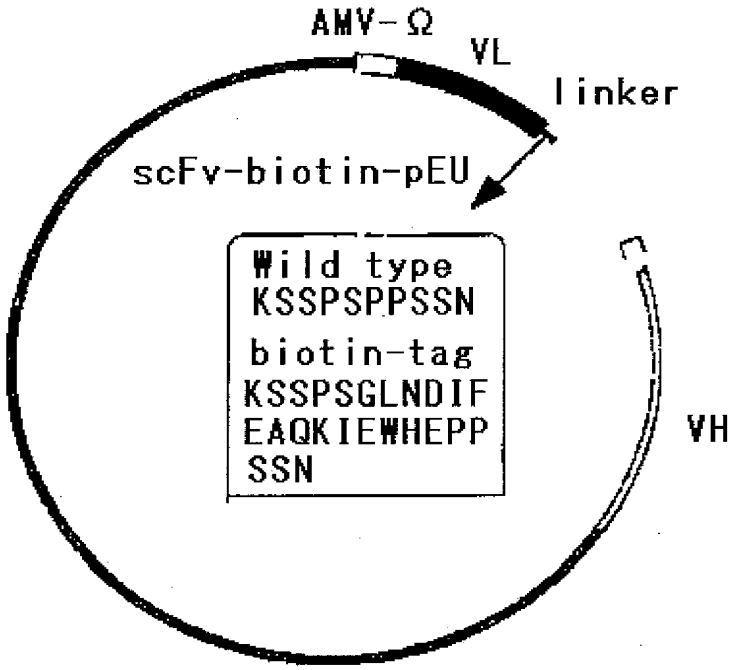
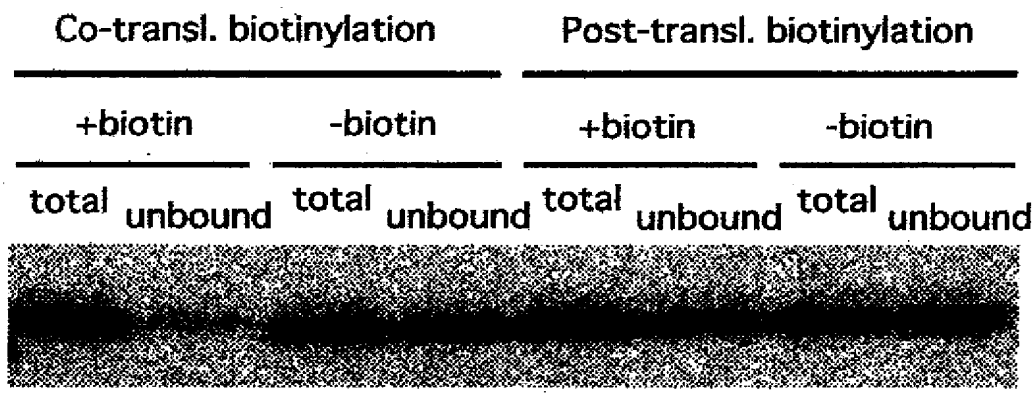


Fig.2


Co-transl. biotinylation
Post-transl. biotinylation
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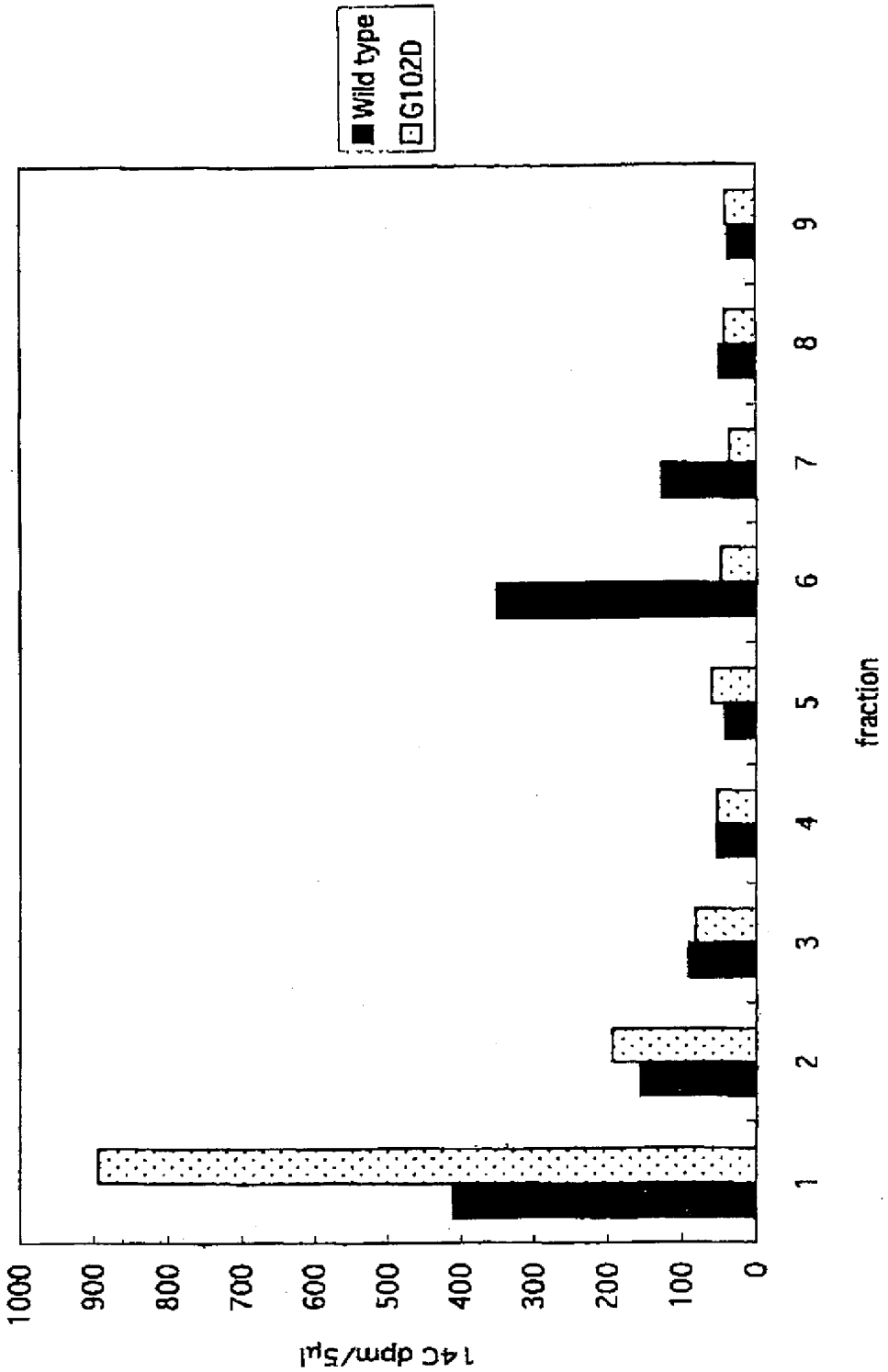
+biotin
-biotin
total
unbound
total
unbound
total
unbound
total
unbound

Fig.3


Wild type
G102D
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900
800
700
600
500
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200
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2
3
4
5
6
7
8
9
fraction

Fig.4

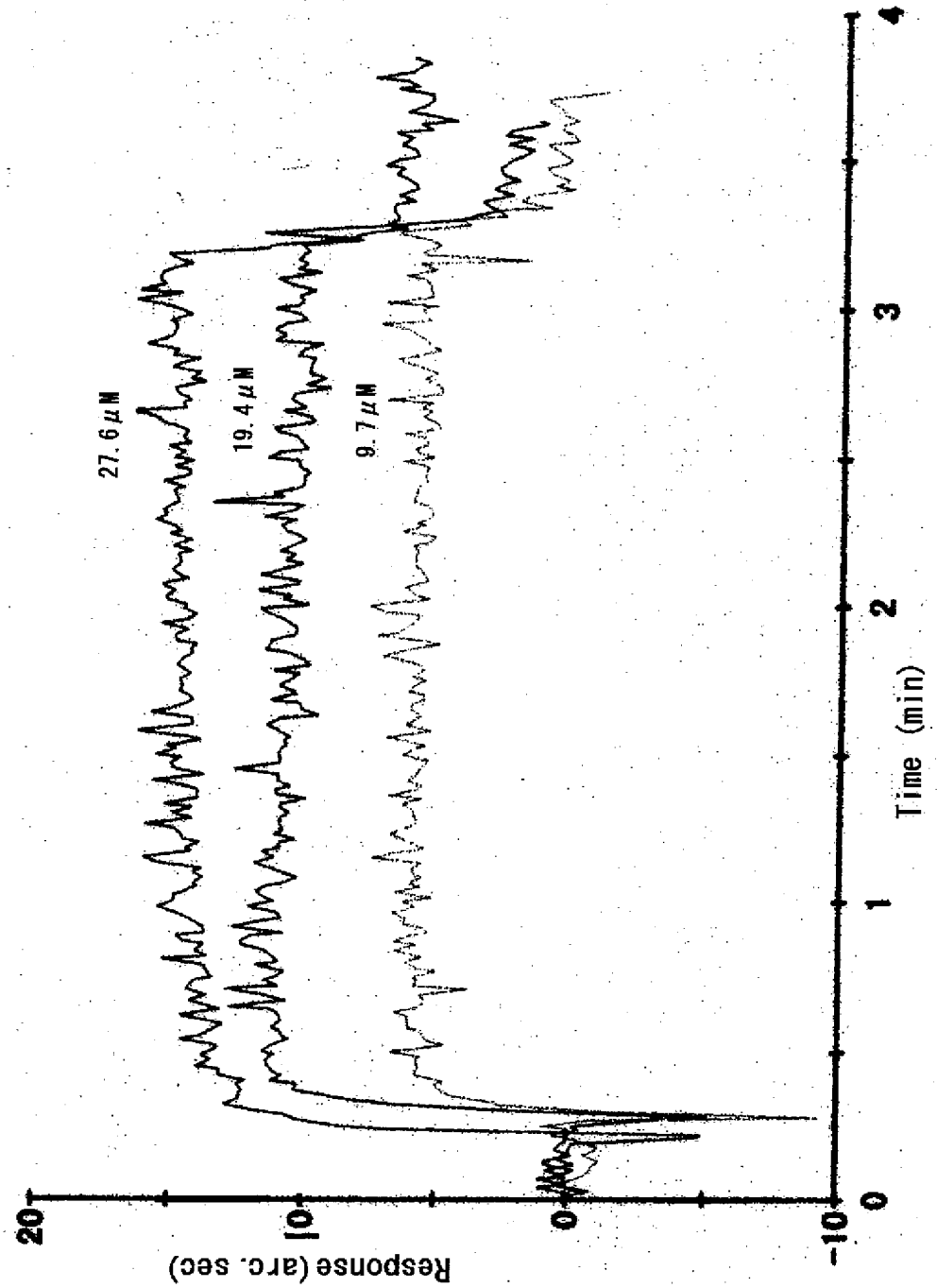
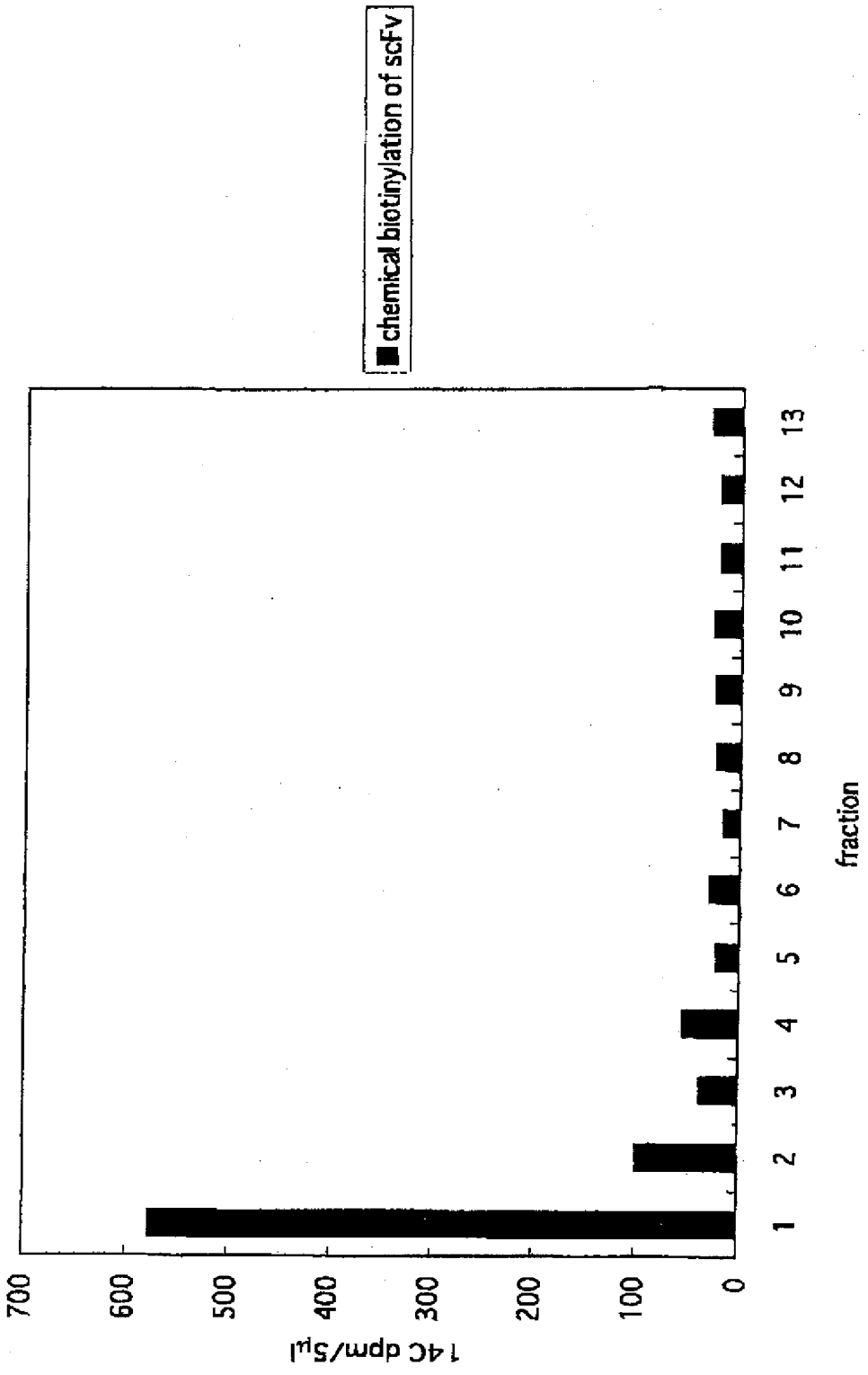


Fig.5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100
200
300
400
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600
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2
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4
5
6
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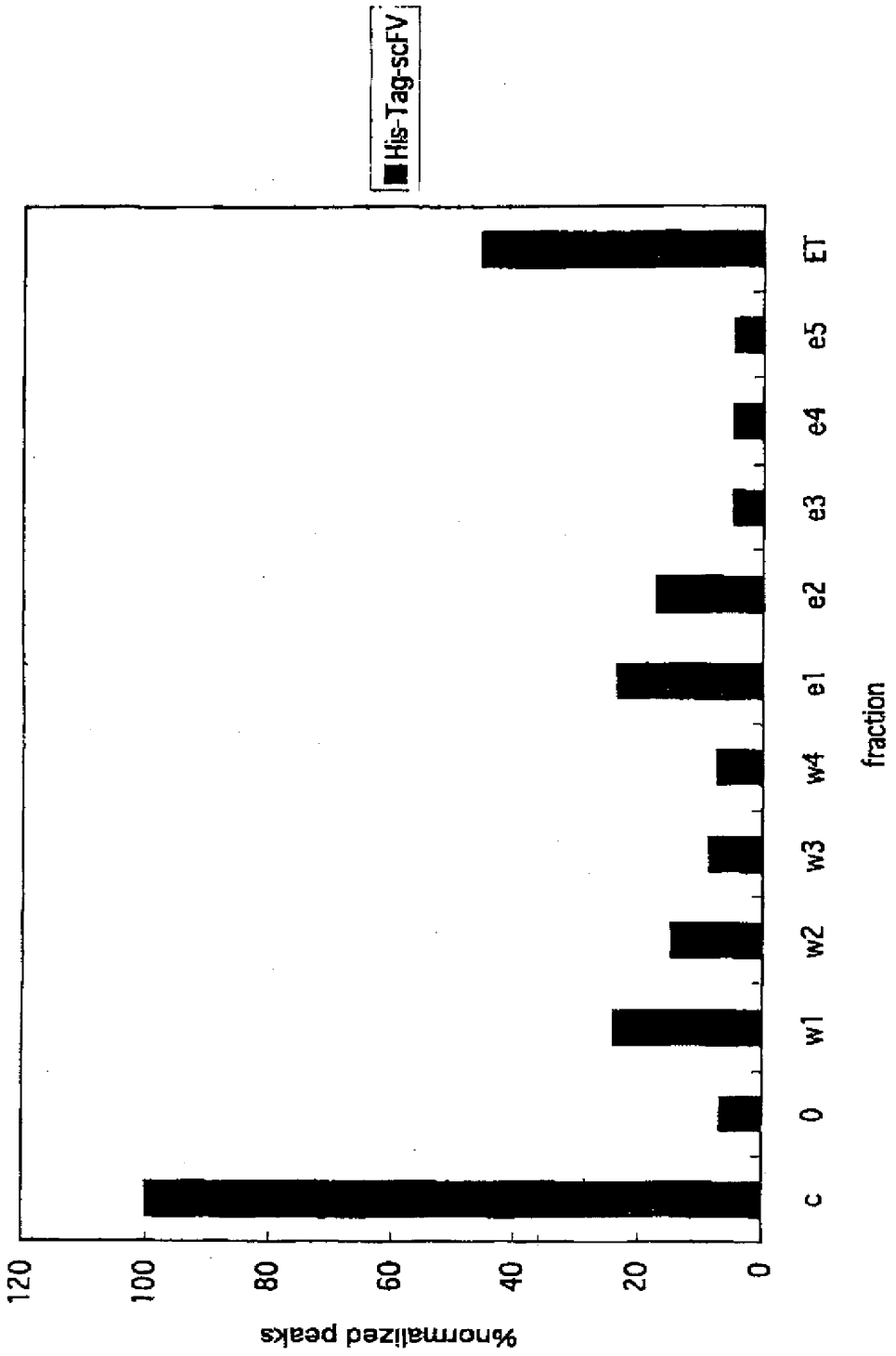
9
10
11
12
13
fraction

Fig.6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%normalized peaks
120
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80
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c
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w1
w2
w3
w4
e1
e2
e3
e4
e5
ET
fraction

ABSTRACT

The present invention provides a labeled single chain antibody that retains its original specific binding activity with an antigen.

The labeled single chain antibody of the present invention can be produced by linking a labeling substance to a linker part of a single chain antibody. The antibody is produced using a cell-free protein synthesis system, and production is carried out in a low reductive state that allows an intramolecular disulfide bond to be retained. Further, bonding the antibody to a solid phase via the labeling substance enables production of an immobilized single chain antibody as well as a method for analyzing an antigen-antibody reaction using the immobilized single chain antibody.